Chemosensory Control of Filter Feeding Behavior in Bigheaded Carps

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Abstract

Bigheaded carps (silver carp, *Hypophthalmichthys molitrix* and bighead carp, *H. nobilis*) were used to investigate the physiological basis of filter feeding behavior in fish. I developed a new method for assaying chemically based food preferences by tracking the frequency of buccal-pharyngeal pumping behavior before and after a food and food chemical stimuli were presented. Spirulina algae (*Arthrospira spp.*), a cyanobacterium, was the most potent food type in releasing BPP behavior. Quality and quantity of chemical cues were important to the BPP response. Moderate responses to a mixture of L-Amino acids (common fish feeding cues) confirm their function in this genus but also suggest that there are other highly potent odorants and/or tastants present in the food mix filtrate. BPP behavior was markedly reduced without a functional olfactory sense. These results cumulatively suggest that chemical senses are integral to filter feeding behavior of Bigheaded carp.

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Chapter 1: An Introduction

The Bigheaded Carp Problem in the Mississippi River Basin

Aquatic invasive species (AIS) are increasingly being linked to ecological disturbance at a variety of scales and in a variety of ways (Hansen et al., 2013; Parker et al., 2001; Pejchar and Mooney, 2009). Control of AIS populations that have a direct negative impact on humans is a goal with substantial public and state/federal government support. In certain cases the anthropocentric justifications align with conservation biology in the goal of protecting the biodiversity of North American aquatic ecosystems against particularly problematic AIS. Successful ecosystem level reclamations through AIS population control are rare and attempts usually fail due to the lack of effective control methods and problems arising from the economy of necessary scale. Potential control solutions need to be selective and have negligible non-target impacts to warrant use. These requirements make it difficult to engineer control solutions that are viable for large scale implementation.

In the case of the bigheaded carps (the common name referring to two fish species in the genus *Hypophthalmichthys*, also commonly called the "Asian carps", that have been introduced into the Mississippi river basin, invasion is progressing and effective and species specific control methods do not presently exist. Two species of this genus, the silver carp (*Hypophthalmichthys molitrix (Valenciennes 1845))* and the bighead carp (*Hypophthalmichthys nobilis (Richardson 1845))* are the focus of this thesis and are considered a threat to native aquatic biodiversity and ecosystem health because of their wide environmental tolerances, rapid growth, high fecundity, and unique niche

specialization (Cudmore et al., 2012; Herborg, 2007; Kolar and Lodge, 2002; Kolar et al., 2005). Where reproducing populations have become established, these ecosystem engineers modify aquatic food webs by shifting plankton regimes and also compete with native taxa directly (Burke et al., 1986; Irons et al., 2007; Smith, 1993; Williamson and Garvey, 2005; Zhang et al., 2008). Like many successful invaders, bigheaded carps thrive in degraded and channelized river environments, which are ubiquitous in northern temperate regions of the world due to extensive alteration for hydropower, navigation engineering, and other development (Nilsson and Berggren, 2000). Currently bigheaded carps are rapidly dispersing through the Mississippi river basin (Kolar et al., 2005; Wilson, 2014). Silver carp dispersing from the Mississippi river are known to be established as far north as Iowa, as far west as Kansas, and as far east as Ohio. Bighead carp dispersing from the Mississippi river are known to be established as far north as South Dakota, as far west as Colorado, and as far east as Ohio. Adults of both species have been collected farther upstream from these established populations but are not yet abundant (nas.er.usgs.gov 4/13/2015).

There is currently a great demand for effective and species specific control methods that target the bigheaded carps (Kolar et al., 2005). Along with other promising options to manipulate fish behavior in a species-selective manner (aversive sound stimuli and pheromonal attractants), the use of chemical feeding attractants that mimic preferred food types could be a very powerful way to manipulate distributions of feral bigheaded carps. This introductory chapter will introduce the peer-reviewed literature relevant to this topic and build a case for applied research in a narrative manner from basic biology

to current control efforts. It is hypothesized that filter feeding behaviors of these fishes present a weakness that can be exploited with chemical stimuli found in the natural chemical feeding cues of phytoplankton. The ability to control behavior and spatial distribution of these fishes in a selective manner would in theory enhance control methods.

Bigheaded Carp Life history, Morphology, Ecology, and Invasion Biology

The genus *Hypophthalmichthys*, literally translated "under eye fish", was first described by Bleeker (1860). Recent analyses confirm that the genus *Hypophthalmichthys* is valid (Howes, 1981; Li et al., 2009; Tang et al., 2013). There are three species described in the genus: the silver carp, the bighead carp, and the largescale silver carp (*Hypophthalmichthys harmandi* (Sauvage 1884)). The silver carp and the bighead carp were introduced in North America for biocontrol of nuisance phytoplankton in aquaculture ponds and wastewater treatment systems (Kolar et al., 2005).

Life history characteristics of bigheaded carps are reviewed in detail by Kolar and others (2005). Briefly, spawning typically occurs in areas of turbulent flow (Duane Chapman Pers. Comm.). Fertilized eggs become neutrally buoyant and drift downstream until they hatch as larvae after about 30 hours (Chapman and George, 2011). Larvae continue to drift downstream until they develop into free swimming fry, after which very little is known about where they go and what they eat (Kolar et al., 2005). Long stretches of unimpeded river are optimal for development, but these requirements seem to be more flexible in introduced ranges (Coulter et al., 2013). Adult bigheaded carps grow fast and

can mature after only 2 years (Kolar et al., 2005; Williamson and Garvey, 2005; Zhou et al., 2009). Adult female bigheaded carp are highly fecund and a gravid individual can produce upwards of a million eggs (Kolar et al., 2005).

The genus *Hypophthalmichthys* is characterized by; a stout body, large head, massive opercles with relief structures, head and opercles scaleless, gill membranes broadly joined across the isthmus, snout bluntly rounded, mouth terminal with thin lips, lower jaw slightly protruding, barbels absent, and jaws without teeth. The eye is small, located far forward below angle of the jaw, and projects downward. Scales are small, cycloid, and cover the entire body; and lateral line is complete. The dorsal fin originates posterior to the pelvic fin insertion, typically has fewer than nine branched rays and lacks an osseous spine. The anal fin typically has more than 10 branched rays. Pharyngeal teeth are typically in one row, four on each side, masticatory surface sole-shaped. The intestine is long and convoluted (Kolar et al., 2005).

Two defining morphological features of the genus are the gill rakers and epibranchial organ. Silver carp gill rakers are bifurcated and fused together with a very fine inter-gill raker distance, while bighead carp have gill rakers in similar configuration but without fusiform structures and a slightly larger inter-gill raker distance (Boulenger, 1901; Fang, 1928). The epibranchial organ of silver and bighead carp are similar and have internal lumens and elongated palatal folds associated directly with opposing gill rakers (Hansen et al., 2014; Wilamovski, 1972). Epibranchial organs are present in many fish taxa, are always associated with microphagy, and facilitate filtration of micro-particulate food from the environment (Hansen et al., 2014; Lazzaro, 1987; Nelson,

1967). Mucus secretion from the epibranchial organ is likely important for packaging of micro-particulate foods for ingestion (Hansen et al., 2014). The outer surface of the epibranchial organ of the bigheaded carps is known to sense chemical food cues and may modulate reflexive filter feeding behaviors (discussed in detail below). The internal spaces within the epibranchial organ are also gustatory and likely function as part of an accessory pumping mechanism that enhances filtration (Hansen et al., 2014; Wilamovski, 1972).

The specialized morphology and feeding mechanics of the bigheaded carps have prompted substantial research studying their diets. Most of these studies employ the quantification of gut contents and calculation of electivity indices for fish from wild and captive populations (reviewed in detail by Kolar et al., 2005). Studies of gut contents and electivity generally concur that both silver and bighead carp consume particulate foods of many types in the 10-1000 micrometer range at all life stages: including phytoplankton, zooplankton, plant pollen, detritus, insect larvae, bacterial coliforms, and incidental sediment (Cremer and Smitherman, 1980; Dong and Li, 1994; Kolar et al., 2005; Spataru and Gophen, 1985; Vörös et al., 1997). Stable isotope analyses confirm the low relative trophic level of these fishes, consistent with studies of gut contents (Chen et al., 2010; Li et al., 2013; Zhou et al., 2009). Few clear patterns emerge from electivity indices suggesting that either diets are not selective and highly opportunistic, that diet preferences are highly variable and context specific, or that selection only occurs via the combination of passive mechanical filtration function (selects for larger particles) and behavioral selection of feeding area (Dong and Li, 1994; Smith, 1989). The gut contents

of wild bigheaded carps in the Mississippi River usually reflect the composition of food items present in the water column where they are captured (low electivities), and cases of high electivity are usually associated with a food type that is spatially stratified in the environment such as floating colonies of the cyanobacterium *Microcystis aeruginosa* (Duane Chapman Pers. Comm.). It has been generally concluded that species in the genus specialize on plankton predation at all life stages and exhibit diet/niche overlap. Consensus exists in that the bighead carp tends toward zooplankivory while the silver carp is predominantly phytoplanktivorous (Kolar et al., 2005). It is known that silver and bighead carp actively feed on and efficiently assimilate energy from cyanobacteria (even taxa that can produce defensive toxins), which is a rarity among vertebrate taxa (Beveridge, 1993; Kolar et al., 2005; Ye et al., 2013). Cyanobacteria are a lineage of photoautotrophic single celled organisms that are abundant in lakes and rivers, express unique photopigments, and produce unique suites of chemical cues at concentrations that could be biologically active as infochemicals (Jüttner, 1995; Watson, 2003). In keeping with the applied goal of species selective control, the apparent open niche occupancy cannot be overlooked as a potential avenue of species selectivity.

Four East Asian carp species: the bigheaded carps (silver and bighead), grass carp *Ctenopharygedon idella* (Valenciennes1844), and the Black carp *Mylopharyngedon picea* (Richardson 1846)) have been intentionally introduced in many places outside of their native range for two reasons. First, they are excellent species for aquaculture production (Michielsens and Lorenzen, 2002). Second, in North America it was hypothesized that these fishes can serve as a biocontrol agent for nuisance phytoplankton, plant, and

gastropod populations (Kolar et al., 2005; Smith, 1993; Zhang et al., 2008). Bigheaded carps were thought to be an ideal biocontrol agent for these nuisance phytoplankton species, which often become significant problems for water quality and various industrial pursuits such as intensive aquaculture of catfish and human wastewater treatment (Zhang et al., 2008). Introductions occurred all over the world and led to several established populations of which multiple are now viewed as invasive (Kolar et al., 2005). Considerable effort was expended in the fields of community ecology, biomanipulation, and aquaculture to measure the effects of these introductions, usually biased towards demonstrating a positive biocontrol outcome.

D. W. Smith (1989) was the first to directly and objectively test the question of whether these fish were actually able to selectively filter feed and thus deliver the predicted biocontrol outcomes. Controlled lab scale experimentation eventually revealed that filter function was a passive, mechanical function of filter morphology, not particle selection (Cremer and Smitherman, 1980; Dong and Li, 1994; Smith, 1989). Cross-flow filter function of the teleost buccal and pharyngeal cavity supports this conclusion (Brainerd, 2001; Sanderson et al., 2001; Silberberg and Segre, 1962; Smith and Sanderson, 2008). A cross-flow filter works by utilizing the tendency of suspended particles to resist adduction when in solution flowing parallel to the plane of filter pores. The silver and bighead carp have a large cross-flow filter surface area comprised of the epibranchial organ and associated gill rakers and can consume particles smaller than their inter-gill rake distances (Fang, 1928; Hansen et al., 2014), further implicating cross-flow filter function. Limited particle selection abilities mean that spatial selection of feeding

area and tight modulation of feeding behaviors via chemosensory perception is important for these fish (Dong and Li, 1994).

Many introductions of bigheaded carps have been successful at producing biomass for food production but have failed to produce the intended biomanipulation goals in the long term (Smith, 1993; Zhang et al., 2008). The reasons behind these failures are complex and often context specific, but the most parsimonious explanation is derived from clear trends in size-selective particle filtration (Vörös et al., 1997). Silver and bighead carp are better at filtering particles that are on the larger end of their diet size spectrum (consistent with cross flow filter function). This fundamental aspect of bigheaded carp feeding biology complicates biocontrol of phytoplankton because they cannot effectively filter picophytoplankton, which become dominant when released from predation by zooplankton and competition for nutrients with macrophytoplankton (Smith, 1993; Zhang et al., 2008). Size selective phytoplanktivory triggers trophic cascade in most systems upon introduction of bigheaded carp (eutrophic to oligotrophic, not hypereutrophic) often resulting in a regime shift in plankton assemblage towards smaller forms along with a constant or increased total phytoplankton biomass (Smith, 1993; Zhang et al., 2008).

There are at least four primary reasons why bigheaded carps are a potential threat to indigenous fauna of North America or elsewhere: (1) they are specialized (filling the ecological niche of microphagy) and exist at a low trophic level, (2) they grow extremely fast and can reach extremely high abundances, (3) they are highly fecund, and (4) they are ecosystem engineers. Competitive interactions with native fishes are documented, but

the existence of these interactions depends on the existence of a growth limiting supply of planktonic food. Sampson and others concluded that direct competition would exist in the Mississippi River between native genera exhibiting diet overlap with invasive *Hypophthalmichthys* such as *Dorosoma*, *Polyodon*, and *Ictiobus* (Sampson et al., 2008). Condition factors of *Ictiobus* (buffalo) and *Dorosoma* (gizzard shad) in the Mississippi river were found to be negatively related to the catch rates of bigheaded carp (Irons et al., 2007). To date no robust research to date has been able to demonstrate that plankton are a limiting resource to fishes in the Mississippi River, while the abundance and diversity of the plankton community has been described thoroughly at many scales (Baker and Baker, 1979; Baker and Baker, 1981; Lair, 2006; Søballe and Kimmel, 1987). Regime shifts in plankton communities after introduction of bigheaded carps could also indirectly impact native taxa.

Bigheaded Carp Population Genetics and Anthropogenic Hybridization

In addition to basic biology and ecology, a brief review of what is known about the population genetics of the bigheaded carps is relevant in order to fully understand the genus of focus in the context of their introduced range in the Mississippi River basin. Synthesis of theory and interpretation of data from these fields can explain why these fish are uniquely invasive, inform risk assessments, and lend insight into achieving applied goals.

Production of fertile hybrids from silver and bighead carp is well documented. The production of fertile hybrids facilitates introgression in some introduced populations

of silver and bighead carp (Lamer and Dolan, 2010). It has been postulated that the mechanism of reproductive separation is partially or completely broken in the Mississippi introduced range, as rates of hybridization appear to be lower in native ranges in Asia (Kolar et al., 2005; Lamer et al., 2013). Furthermore, significant genetic divergence of invasive populations from source populations has been documented using mitochondrial DNA markers (Li et al., 2011). The question remains whether the genetic differentiation that has been observed is due to neutral population drift or a rapid evolutionary response to a novel environment caused by humans.

Anthropogenic introduction of a species into a novel environment creates the potential for a rapid evolutionary response both in the invader and the invaded ecosystem (Parker et al., 2001). Hybrid speciation has been linked to the introduction of congeners into novel environments in the cyprinid *Gila* species complex (natural geological event) and in the teleost genus *Cottus* (anthropogenic event)(DeMarais et al., 1992; Nolte et al., 2005). Furthermore, mechanisms of animal evolution involving homoploid hybrid hybridization (HHS) are likely more prevalent in animal taxa than previously thought (Mallet, 2007). HHS is defined as hybridization without polyploidy between two closely related species, and can give rise to lineages quite different from parental lines (Gross and Rieseberg, 2005; Mallet, 2007). In the case of the introduced populations of bigheaded carps, it is clear that the basic requirements for occurrence of anthropogenic HHS are met. The bigheaded carp metapopulation in the Mississippi River system has substantially destabilized into a hybrid swarm (Lamer and Dolan, 2010). Acceleration of

evolutionary processes in response to anthropogenic selection pressures may partially explain why this particular invasion has been so exceptionally rapid and persistent.

Consideration of the divergent evolutionary trajectory of introduced bigheaded carp has several implications for research and development of control methods. Risk assessments for these and other introduced species should consider this factor in making ecological and spatial predictions. At this time research on control methods should be focused at the genus level due to ambiguity and uncertainty of species level differences using feral fish from the Mississippi River population. Caution must be exercised when comparing silver and bighead carp behavior and physiology and when comparing bigheaded carps between native and introduced ranges (Duane Chapman Pers. Communication). Doing so may result in incorrect assumptions, as evidenced by the unexpected plasticity in spawning requirements of these species that has been observed in North America (Coulter et al., 2013).

Neurobiology and Function of Chemosensory Systems of Fish

A review of relevant literature pertaining to the neurobiology and function of food chemosensory systems of fish is warranted given the intimate role of chemically mediated behaviors in postulated control regimes for many fishes. Chemical sensitivity is relatively broad in fish olfactory systems (Derby and Sorensen, 2008; Kotrschal et al., 1998). Chemical senses are important to fish living in highly turbid environments such as large rivers (Derby and Sorensen, 2008; Kotrschal, 2000; Sorensen and Caprio, 1997). Three model teleost species presently serve as the basis of the current understanding of

chemoreception in fish: the goldfish (*Carassius auratus*), the channel catfish (*Ictalurus punctatus*), and the rainbow trout (*Onchorynchus mykiss*). Four functionally distinct types of chemosensory systems model have been described in teleost fishes: olfaction (cranial nerve I), gustation (cranial nerves VII, IX, X), trigeminal (cranial nerve X), and solitary chemosensory cells (not known to be associated with any particular cranial nerves)(Derby and Sorensen, 2008; Hansen, 2007; Kotrschal, 2000; Sorensen and Caprio, 1997; Yamashita et al., 2006). The neurobiology, physiology, and functional roles of olfaction and gustation are relatively well defined and will be briefly reviewed. The trigeminal sense and the solitary chemosensory cell system are poorly understood in fish, and will not be reviewed (Hansen, 2007; Sorensen and Caprio, 1997). It is often concluded that olfaction and gustation function in a synergistic manner in the case of mediating feeding behaviors and possibly other behavioral functions (Derby and Sorensen, 2008; Kotrschal, 2000; Sorensen and Caprio, 1997).

The olfactory sense of fish is homologous to smell in humans, being highly conserved throughout vertebrate evolution. Olfactory systems of fish are known to be sensitive to several classes of water soluble odorants (amino acids, nucleotides, polyamines, bile acids, gonadal steroids, and prostaglandins) at low concentrations [picoMolar to microMolar] (Derby and Sorensen, 2008; Hansen and Zeiske, 1993; Laberge and Hara, 2001; Sorensen and Caprio, 1997). The sensory epithelium comprises the peripheral olfactory organ, which is found in a specialized cavity that allows controlled water flow over the sensory epithelium (olfactory nare). It contains sensory and non-sensory regions and forms a complex ultrastructure called the olfactory rosette.

There are three morphological types of olfactory receptor neurons (ORNS - Ciliated, Microvillous, and Crypt) in the olfactory epithelium. These are attributed to primary afferent functions of the olfactory sense. Each ORN contains a single type of receptor protein; gene products of which the diversity, sensitivity, and function are poorly understood (Sorensen and Caprio, 1997). ORNs are continuously replaced through the life of the organism by stem cells (Kotrschal, 2000). Most ORNs convert receptor binding events into electrical impulses through second messenger systems that include G-Proteins. ORNs are the neurons of cranial nerve I, whose axons project directly to the olfactory bulb. The olfactory bulb is made up of intermediate processing nodes called glomeruli, which often contain several types of integrative neurons in fish (mitral, granular and ruffed). All ORNs with common receptors appear to converge on specific glomeruli, creating a spatial code in the brain and way that signals can be amplified (Sorensen and Caprio, 1997). Mitral neurons directly synapse with and integrate multiple afferent ORN inputs. Precise mechanisms of odor processing are not well understood, but evidence of complex spatial organization and temporal coding of neural activity exists in goldfish and salmonids. In the goldfish, the medial and lateral mitral projections into the telencephalon seem to mediate pheromonal and food functions respectively (Sorensen and Caprio, 1997).

The gustatory sense of fish is homologous to taste in humans, also sensing water soluble chemicals. Gustatory systems usually detect relatively high concentrations [microMolar to milliMolar] of tastants relative to the olfactory system (Kotrschal, 2000; Sorensen and Caprio, 1997). Nucleotides, quaternary ammonium compounds, and

organic acids are known to stimulate gustatory neural activity in various taxa (Sorensen and Caprio, 1997). The gustatory sense is thought to primarily mediate reflexive and close range feeding behaviors, and can be viewed as an array of specialized sub-systems. Taste buds are comprised of 30-100 specialized epithelial cells that form synapses with gustatory nerve fibers and are the peripheral sensory units of the gustatory system. (Sorensen and Caprio, 1997). Taste buds are usually found only inside the oral cavity or on the lips, but in catfish a unique extra-oral gustatory field is present along with typical intra oral taste fields (Kanwal and Caprio, 1983; Valentincic and Caprio, 1994). Gustatory sensory fields containing taste buds are innervated by the 7th, 9th, or 10th cranial nerves (Finger, 2009; Kotrschal, 2000; Sorensen and Caprio, 1997). L-Amino acids are the primary class of tastants commonly associated with reflexive ingestion behaviors (Michel and Caprio, 1991; Valentincic and Caprio, 1994). In all instances studied, taste systems are tuned to respective feeding niches of their hosts (Derby and Sorensen, 2008). Recent research on bigheaded carps has documented the existence of a pharyngeal taste system innervated by cranial nerve 10 and associated with the epibranchial organ that responds to food homogenate and L-Amino acids. Buccal taste fields were small in these species (Hansen et al., 2014).

Further research is needed to describe and characterize the function of the chemosensory systems of microphagous fishes, including the bigheaded carps, especially sensitivities to novel types of tastants/odorants and their relative roles in feeding behaviors. This research should include histological, electrophysiological, and behavioral analyses of bigheaded carps from populations of interest for control. Proper

considerations of neuro and population biology should be taken (see section III). There is a large body of existing literature documenting chemical species that are associated with dense populations of phytoplankton that could be tested in purified form for physiological and/or behavioral activity (Fink et al., 2006; Jüttner, 1995; Watson, 2003). It is highly likely that many of these chemical species function as chemical food cues for bigheaded carps due to their unique diet specialization. A complementary approach called bioassay guided fractionation could be used to find chemical cues of preferred foods that have not been previously described. This method would start via the characterization of preferred genera/species of phytoplankton. This would be followed by isolation of the behaviorally active chemical species they release using behavioral assays in combination with advanced biochemical fractionation techniques. Comparisons of physiological and behavioral activity produced by chemical cues of phytoplankton can then be used to produce a maximally potent attractant or stimulant of feeding behavior.

Chemosensory Feeding Behavior of Bigheaded Carps

The feeding related ethogram of the bigheaded carps is only partially described, and the chemosensory mediated behavior and physiological tuning of the genus are completely undescribed except for recent work on the epibranchial taste system. An integration of the epibranchial organ, gill rakers, and the pharyngeal teeth composes the microphagous feeding system (Hansen et al., 2014). Precise functional mechanisms of this type of filtration apparatus are unknown, but the distinctive feeding behavior employed by fishes possessing epibranchial organs has been termed filter-feeding, pump

filtering, buccal pumping, buccal/opercular pumping by various authors (Dong and Li, 1994; Kolar et al., 2005; Lazzaro, 1987; Smith, 1989). Due to ambiguity of previous terminology the term buccal-pharyngeal pumping will be used herein because it is most comprehensive. Buccal-pharyngeal pumping (BPP) behavior is used for respiratory function in all jawed fishes that have not adapted to using ram-filtration (requires constant locomotion). A secondary function of this pump mechanism has evolved (an exaptation) to facilitate suction and filter feeding. Filter feeding and is similar among many known microphagous fishes; consisting of the buccal-pharyngeal pump mechanism used in a rhythmic action (~ 2.5 Hz) to aggregate food particles in conjunction with respiratory functions (Lazzaro, 1987; Zhao et al., 2011). In the silver carp, BPP behavior rate has been shown to be modulated by the effects of dissolved oxygen level, temperature, food particle type and density, and by the presence of toxic cyanobacteria in the environment as shown by specific lab scale experiments (Beveridge, 1993; Dong and Li, 1994; Zhao et al., 2011; Zhao et al., 2014). Diets of bigheaded carps are well established through studies of gut contents, but little is known about the physiological basis of BPP behavior, food selection, and related higher order behaviors such as attraction, pattern recognition, and conditioning. Habitat selection by silver carp has been linked to food resources using telemetry in the Mississippi River (Calkins et al., 2012). Habitat selection patterns break down in periods of high flow, which are also correlated with dispersal patterns and spawning aggregations (DeGrandchamp et al., 2008; Kolar et al., 2005). While supporting information is limited, it appears that movement is an important component of foraging behavior for the bigheaded carps in the dynamic large

river environment. This is an important piece of information for potential control efforts utilizing chemical food attractants because it suggests that they could work at large spatial scales.

The relative roles of gustation and olfaction in control of feeding behavior have been elucidated in only a few model fish species. Where studied in goldfish, rainbow trout, and channel catfish; olfaction is concluded to have a salient role in mediating complicated, conditioned, or conditional behaviors such as arousal, learning, pattern recognition, coordinated spawning, and attraction behaviors (Derby and Sorensen, 2008; Laberge and Hara, 2001; Sorensen and Caprio, 1997). The associations and behaviors derived from olfactory stimulation tend to be plastic, and ORNs are known to be continuously regenerated over time. Conversely, the close range detection, selection, and ingestion of food are primarily controlled by gustatory reflex loops in the hindbrain collectively responding to sensation of chemical food cues and/or other stimuli inside the oral cavity (Derby and Sorensen, 2008; Sorensen and Caprio, 1997).

Fish, like many animal taxa, often use available sensory information to continuously sample their environment and inform behavior (Emde and Mogdans, 2004). The degree to which one or the other sensory systems affects higher order behavior can potentially vary between taxa, ecological niches, genetics, context, and other factors. Although functional separation can occur, synergistic action of two or more sensory modalities in creating perception and control of higher order behavior is also common (Filingeri et al., 2014; Kotrschal, 2000). In the case of the bigheaded carps, little is known about which senses are involved in filter feeding behavior. The generalized

chemosensory feeding model derived from the study of model teleost fishes is the best framework for characterizing the chemically mediated filter feeding behaviors of bigheaded carps. In this model, chemically mediated feeding behavior is divided into four distinct phases; recognition/perception of food, attraction to food source, sampling of food source, and finally ingestion/rejection of the food item (Derby and Sorensen, 2008; Sorensen and Caprio, 1997). A stepwise description of the generalized chemosensory feeding model follows:

Recognition/Perception: In a hypothetical context, relatively low concentrations of chemical cues released by food particles are initially sensed by the olfactory system and cue the fish to likely presence of food; this causes a characteristic search behavior to be released if cues are appropriate.

Attraction/Search: Swimming activity increases after detection of appropriate chemical cues. Some fishes appear to be able to find sources of chemical cues by changing their swimming speed (chemo-orthokinesis), by changing turning frequency (chemo-klinokinesis), and/or by following concentration gradients (chemotaxis). If the fish moves into an area where food particles are present in adequate concentration, swimming activity ceases and sampling behavior is released.

Sampling: When the chemosensory systems sense the presence of food in the immediate range of the primary mode of feeding (directly in front of the mouth, in the field of vision, or touching the body); potential food items are brought into the buccal-pharyngeal cavity by BPP behavior where the intra-oral gustatory subsystem likely assesses identity and palatability.

Ingestion/Rejection: If the food item is palatable, the swallowing reflex and the action of smooth muscle in the esophagus transport the food item(s) into the alimentary canal to be digested. If the food item is unpalatable, reverse BPP behavior expels the food from the buccal-pharyngeal cavity.

Successful execution of this sequence of feeding behaviors is important for survival and thus represents a primary weakness to exploit in the context of control. Focused study of the feeding behaviors of bigheaded carps and comparison with the general model is an important first step in developing control methods. Morphological peculiarities of the bigheaded carps may define departures from the generalized feeding behavior model described above (Hansen et al., 2014).

Current Control and Development of Novel Methods

Strong public support has been garnered for finding a solution to the bigheaded carp problem due to the great angst caused by these particular fish. Currently many options are being explored for managing populations of bigheaded carps including use of percussive sound cannons to kill and direct fish, rapid response seine netting for positive eDNA detections for bigheaded carps in water samples, bow/spear/net fishing tournaments, and the development of commercial markets for these fish domestically and abroad. While encouraging, these methods have not yet proven effective.

Attempts at limiting dispersal through installation of permanent physical, electric, and sound and light barriers at strategic places are underway in the Mississippi River basin. While limiting dispersal is very important, it cannot contribute to control of

populations already established or that may become established in the future: highlighting the future need for effective control methods. A large commercial fishing program is currently financed by the state of Illinois and the United States Fish and Wildlife Service in an attempt to combat the exponential growth in abundance and rapid dispersal observed (Sass et al., 2009). Unfortunately this approach suffers from the fact that these fish are currently difficult to capture with conventional commercial fishing methods, their high fecundity and apparent density dependent reproduction, and economies of scale. Population model projections based on the critical population parameters of the Illinois river bigheaded carp populations suggest that fishery induced collapse will not occur at current harvest levels and/or if harvest remains size selective for large adults (Tsehaye et al., 2013). Development of methods for targeted poisoning with Antimycin (Fintrol) based nano-piscicide is currently underway by the USGS using a species specific vector mechanism (Rach et al., 2009). Currently there are no capture methods being employed or being developed to specifically target the juvenile life stages of these fishes.

To achieve the goal of population management using novel methods that target life history weaknesses, assumptions about sensory mediation of these behaviors and associative learning capabilities of bigheaded carps must be experimentally validated in a robust manner at lab and then field scales. Special attention should be given to potential weaknesses related to bigheaded carp feeding behavior that could be exploited to enhance current control methods. For example, a potential use of chemical attractants and feeding stimulants could be to add efficiency and another layer of selectivity to nanoparticle based piscicides currently under development by the USGS Upper Midwest

Environmental Science Center. Another potential use would be to manipulate distributions of feral bigheaded carps to enhance eDNA monitoring and/or commercial netting efforts. For best results of any potential control method, the associative learning abilities of the bigheaded carps should also be characterized and exploited. Functional demonstration of behavioral manipulation with chemical food cues in the lab and in the field is needed to garner support for use of such methods, which could lead to use at an effective scale. Ultimately, any control methods must be at least genus specific for bigheaded carps and environmentally safe in order to properly achieve a sustainable outcome.

Hypothesis Statements and Research Questions

Bigheaded carps detect unique suites of chemical cues associated with preferred food types and use them to efficiently forage, contributing to their invasiveness. Feeding behaviors of bigheaded carp are stimulated on a large scale with these chemical cues. Primary Research Questions (Chapter 2):

1.) Is BPP behavior rate stimulated by the presence of food?

2.) Are BPP behaviors of the bigheaded carps chemically mediated?

3.) Do the bigheaded carps have chemically based diet preferences?

4.) Does quality or quantity of chemical food cues modulate BPP behavior?

5.) Do common amino acids explain the chemically mediated BPP behavior?

6.) What is the role of the olfactory sense in BPP feeding behavior?

7.) Are there differences between silver and bighead carp?

Chapter 2: Chemosensory Control of Filter Feeding Behavior in Bigheaded Carps

A draft manuscript in preparation for submission to the journal Physiology and Behavior

Summary

Bigheaded carps (silver carp, *Hypophthalmichthys molitrix* and bighead carp, *H. nobilis*) were used to investigate the physiological basis of filter feeding behavior in fish. I developed a new method for assaying chemically based food preferences by tracking the frequency of buccal-pharyngeal pumping behavior before and after a food and food chemical stimuli were presented. The mean respiratory buccal-pharyngeal pumping behavior (BPP) rate for silver and bighead carp over an 8 minute period was 0.09 +/- 0.04 and 0.03 +/- 0.02 Hz (n=12). When a food mixture was presented, mean BPP behavior rate increased to 2.44 +/- 0.25 and 1.97 +/- 0.48 Hz over an 8 minute period and was sustained at an elevated rate for greater than 8 minutes. Chemical components found in the food mixture alone released mean BPP rates of 1.11 +/- 0.59 and 1.15 +/- 0.45 Hz over an 8 minute period for silver and bighead carp respectively. Spirulina algae (*Arthrospira spp.*), a cyanobacterium, was the most potent food type in releasing BPP behavior. Quality and quantity of chemical cues were important to the BPP response. Moderate responses to a mixture of 18 common L-Amino acids (common fish feeding cues) confirm their function in this genus but also suggest that there are other highly potent odorants and/or tastants present in the food mix filtrate. BPP behavior was markedly reduced without a functional olfactory sense. These results cumulatively suggest that chemical senses are integral to filter feeding behaviors of bigheaded carp and that chemical attractants and feeding stimulants have potential to enhance current control methods for this genus.

Introduction

Microphagy, or specialization for the consumption of micro-particulate foods such as phytoplankton and zooplankton (also termed planktivory), is a feeding strategy ubiquitous to the early life stages of all teleost fishes (Gee, 1989; Platt et al., 2003). A subset of teleost taxa continues to use this feeding mode through later stages in their life history, with a subset of these exhibiting microphagy through their entire lifespan. These fishes are globally distributed in marine and freshwater, where microphagous species often play important ecological roles through occupying relatively low trophic levels and are commercially important to human society as a source of protein (Lazzaro, 1987). Feeding at a lower trophic level allows fishes to use a larger fraction of the energy produced by primary production. Niche colonization and specialization by natural selection has produced pronounced anatomical and behavioral adaptations/exaptations for consuming microscopic food items (Bauchot et al., 1993; Hansen et al., 2014; Lazzaro, 1987). Very little empirical evidence about how microphagous fish detect and orient to food sources exists, but vision is thought to be of limited utility for feeding on extremely small food particles in turbid environments (Brooks and Dodson, 1965; Derby and Sorensen, 2008). In this study we investigated how chemical food cues control the feeding behaviors of two microphagous species, thereby providing insight into their chemical ecology.

The silver (*Hypophthalmichthys molitrix*) and bighead (*H. nobilis*) carp are native to eastern Asian rivers and lakes and are currently colonizing the Mississippi drainage basin (Kolar et al., 2005; Tang et al., 2013; Wilson, 2014). In their native range these fish

are prized for human consumption while in introduced ranges they are often seen as a nuisance species threatening the welfare of humans and indigenous fauna. Great effort is being expended on management of these species. Fish of the genus *Hypophthalmichthys*, translated "under eye fish", are cypriniform fishes with a unique buccal-pharyngeal morphology and large down-set eyes (Fang, 1928; Hansen et al., 2014; Kolar et al., 2005). The genus has been studied for use in biocontrol because its members specialize in consuming plankton. They thrive in large productive river systems from the tropics to the sub-arctic, and in some environments can cause regime shifts in plankton communities through trophic cascade (Kolar et al., 2005; Smith, 1993).

Bigheaded carps can grow fast consuming a wide array of micro-particulate foods such as zooplankton, phytoplankton (including cyanobacteria), pollen, bacteria, and detritus of various sources determined by studying gut contents (Cremer and Smitherman, 1980; Dong and Li, 1994; Pongruktham et al., 2010; Radke and Kahl, 2002; Spataru and Gophen, 1985; Wilamovski, 1972; Williamson and Garvey, 2005; Xie, 2001). Food selection is likely but very difficult to quantify (Lazzaro, 1987; O'Brien and Vinyard, 1974). Carbon labelling techniques confirm that silver and bighead carp feed at an extremely low trophic level and exhibit partial diet overlap (Chen et al., 2010; Li et al., 2013; Zhou et al., 2009). It is the consensus that the smallest particles that can be removed from the environment at a significant rate are about 10μm in diameter, with selection for larger particles occurring as a passive mechanical process (Cremer and Smitherman, 1980; Smith, 1989; Vörös et al., 1997).

The inter gill raker distances of silver carp average about 34 μm and the bighead carp average about 50μm, with enormous surface area created by deep bifurcations (Fang, 1928). This arrangement suggest that the gill rakers may form a biological cross-flow filter feeding apparatus (Sanderson et al., 2001). In the case of the bigheaded carps, an integration of the epibranchial organ, gill rakers, and the pharyngeal teeth composes the microphagous filter feeding system (Hansen et al., 2014). Precise functional mechanisms of this type of filtration apparatus are unknown, but the distinctive filter feeding behavior employed by microphagous fishes possessing epibranchial organs has been termed pump filtering, buccal pumping, buccal/opercular pumping by various authors (Dong and Li, 1994; Kolar et al., 2005; Lazzaro, 1987; Smith, 1989). Due to ambiguity and incomplete definition of previous terminology we will hereafter use the term buccal-pharyngeal pumping (BPP) to describe this behavior because it is more comprehensive and accurate.

BPP behavior is multifunctional; and is associated with respiration and feeding. Both functions occur simultaneously during filter feeding. Respiratory BPP behavior is occurs at a relatively slow rate, but varies depending on dissolved oxygen level and temperature (Zhao et al., 2011). Filter feeding BPP behavior is characterized by an elevated rate of BPP (~2.5 Hz) where studied and has been shown to be modulated by food particle density and presence of toxic cyanobacteria as shown by specific lab scale experiments (Beveridge, 1993; Lazzaro, 1987; Zhao et al., 2011; Zhao et al., 2014).

Little is understood about the sensory biology and chemical ecology of microphagous fishes, and bigheaded carps in particular. The physiological basis of how

they perceive, orient towards, sample, and consume their tiny food items has not been directly studied. As in all teleost fishes, bigheaded carps possess visual, lateralis, auditory, tactile, olfactory, gustatory, common chemical, and solitary chemosensory cell sensory systems (Emde and Mogdans, 2004). Wide variation exists in the physiological basis of behaviors in fishes, as it is driven by evolutionary processes (Kotrschal et al., 1998). Sensory information is integrated in higher order areas of the central nervous system where afferent neural signals from sensory cell subsystems converge to become perception. As in many fishes, perception of naturally occurring chemical stimuli is likely of primary importance for bigheaded carp feeding (Derby and Sorensen, 2008), especially because their food items are microscopic and they live in low-light environments.

In most fishes, feeding behaviors can be separated into 4 components: Perception/Recognition, Attraction/Search, Sampling, and Ingestion/Rejection (Derby and Sorensen, 2008; Valentincic and Caprio, 1994; Valentinčič and Caprio, 1997). The long distance stages of feeding behavior usually involve olfaction and forebrain control: olfaction is ubiquitously important in a wide array of higher order behaviors in animal taxa (Dove, 2015; Laberge and Hara, 2001; Nevitt, 2003; Valentincic and Caprio, 1994). Fish have chemosensory systems adapted for their native habitat, feeding mode, and life history requirements (Derby and Sorensen, 2008; Kotrschal et al., 1998). The physiological sensitivities of fish chemosenses vary widely (Hara, 1994). Work on model cyprinids, the common carp *Cyprinus carpio* and the goldfish *Carassius auratus*, reveals an intra-oral gustatory adaptation that is highly specialized for ingesting food items from

the benthos and is accompanied by an inferior mouth and an enlarged vagal lobe adapted for gustatory palatal/pharyngeal sorting (Finger, 2008; Sibbing et al., 1986). The bigheaded carps lack hypertrophy of the vagal lobe and have a curious superior mouth, yet exhibit many times the palatal/pharyngeal surface area of *Cyprinus spp.* and *Carassius spp*. and feed on particles several orders of magnitude smaller (Hansen et al., 2014). Bigheaded carps possess a complex gustatory epibranchial organ quite different from the typical cypriniform palatal organ. They also possess a well-developed olfactory system that is anatomically typical of cyprinid fishes (Hansen et al., 2014; Andrew Simons and Peter Sorensen Pers. Comm.). When researchers exposed silver carp to non-toxin strains of cyanobacteria they observed increased BPP behavior but did not when exposed to the toxic strains of the same species (Beveridge, 1993). Beveridge (1993) and Smith (1989) concluded that the chemical senses were most likely to be controlling BPP behavior, but did not directly test that hypothesis in their experiments. The relative roles of the senses in control of BPP feeding behavior have not been determined nor have the active chemical cues employed (and specific roles of each) been identified for any microphagous fish.

With the goal of filling knowledge gaps we asked the following questions: 1.) Is BPP behavior rate stimulated by the presence of food? 2.) Are BPP behaviors of the bigheaded carps chemically mediated? 3.) Do the bigheaded carps have chemically based diet preferences? 4.) Does quality or quantity of chemical food cues modulate BPP behavior? 5.) Do common amino acids explain the chemically mediated BPP behavior?

6.) What is the role of the olfactory sense in BPP feeding behavior? and 7.) Are there differences between silver and bighead carp?

Methods

Study Fish, Algal Food Mixture, and Lab Environment

Wild caught (Missouri River) and lab reared silver carp [*Hypophthalmichthys molitrix*] and bighead carp [*Hypophthalmichthys nobilis*] were obtained from the USGS Columbia Environmental Research Center and held in a 40 kiloliter recirculating aquaculture system with well water input of approximately 56 l per min (volumetric turnover time ~12 hours). Groups were sorted to species by phenotype before arrival and were assayed for hybridization (see Appendix 1). The silver carp were 16-25cm in total length and the bighead carp were 5-18cm in total length at the conclusion of experiments. The photoperiod was held constant at (16h:8h). The water temperature was maintained at 21-23 ° C.

Fish were fed a standardized algal food mixture at a rate of 1% body weight per day at ~1700 hours, which resulted in slightly positive bioenergetic balance, slow growth, and manageable waste loads. This algal diet was developed by Robin Calfee (USGS, Columbia, MO) and was comprised of: 19.76 g/L dried spirulina algae (www.bulkfoods.com), 11.4 g/L dried chlorella algae (www.bulkfoods.com), 0.7 g/L Oncor FW™ trout pellet crumble (www.skretting.us), 1.1 g/L tropical flake food (www.aquaticeco.com), 1.64 g/L Otohime C1™ marine larval food (www.reed-mariculture.com), 0.7 g/L nannochloropsis 3600 condensed micro-algal culture, 0.7 g/L

shellfish 1800 condensed micro-algal culture (www.reed-mariculture.com), 0.6 g/L Cyclopeeze™ freeze dried decapod crustaceans (www.argent-labs.com), and 0.6 g/L soluble vitamin mixture (www.aquaticeco.com) in well water (Figure 1; Robin Calfee Pers. Comm.).

General Assay Methods

A feeding behavior assay was developed and used to quantify BPP behavior. General assay methods are followed by experiment specific methods for specific experiments in subsequent subsections. All experiments complied with rules and regulations of the University of Minnesota Institutional Animal Care and Use Committee (protocol 1306-30686A).

Each experiment was scheduled and executed according to a complete or repeated latin square within subject design blocked by replicate tank (12 levels) and test day (varying levels by experiment), with pseudo-randomized time of day nested within test day (3 levels). For each experiment pairs of juvenile silver and bighead carp were transferred into test tanks and acclimatized for a minimum of 30 days. Test tanks were cleaned with a vacuum tube every 14 days. Subject fish were fed algal food mixture once per day (~1700 hours) from above and opposite the viewing pane in a manner that minimizes but cannot eliminate associable auditory, visual, and chemical cues of food presence. Test environments consisted of 70 liter glass aquaria (12), each lit by a single 40 watt incandescent bulb where BPP behaviors of a pair of fish can be observed live or recorded with a video camera. The photoperiod for all tanks was 16h:8h. The input from

the re-circulating system to each tank was 0.5 l per min, with two opposing air stones that create a convection cell. Opaque polycarbonate dividers were placed to visually isolate each tank replicate from the neighboring tanks. Black plastic was used to isolate the tanks from the lab lighting and to create an observation/recording area which utilizes the 1 way mirror effect created by the aquarium glass to visually isolate the fish from observer or camera presence. Great care was taken not to provide visual, auditory or, physical cues while working around the tank array and observing behaviors. These precautions were necessary to ensure candid responses from sensitive bigheaded carps, which exhibited the ability to associate observer presence with food stimuli in pilot experiments.

Chemical test stimuli were prepared from the base food mixture in the same relative concentrations present in the standard algal diet. Ingredients were added to 250ml of well water (from holding facility source) in a centrifuge vial, mixed, and set to rest at room temperature for 1 hour. Stimuli were then centrifuged at 7,000 rpm for 25 min and the filtrate decanted directly into 6 μm paper vacuum filters. The final filtrate was refrigerated until use in Nalgene flasks. All stimuli tested were never greater than four days old. A simple pneumatic delivery system was used to inject test stimuli in way that did not present non-chemical cues (visual, auditory, lateralis) to the fish other than cues contained within the stimulus itself. This was accomplished by positioning the output of the pipette system directly over the airstone upwelling. Before a day's trials began all 12 stimulus reservoirs were filled with 3ml of test stimulus, after which the first trial commenced after a minimum of 30 minutes. A maximum of one trial was performed per day at a pseudo-randomized time on each of (12) experimental replicate tanks. After a

pre-test interval was complete, 3 ml of chemical stimulus was gently injected over a 2 sec interval via the pneumatic stimulus delivery system. The dilution rate of the 3ml stimulus in the test environment was characterized with fluorescein dye trials (Appendix 2).

BPP behavior is readily observable in these conditions. Noldus Observer XT software (Leesburg,VA) was used to score and analyze behavioral data. Observers were not aware of the experimental design schedule or identity of each treatment type to avoid measurement bias. Each observable opening of the mouth was scored as a BPP event and recorded via keystroke with a timestamp. BPP events were summed over 8 min, 4 min, and 30 sec intervals and exported for analysis. Comparing the summed BPP activity over time (8 or 4 minutes) between control and experimental treatments resulted in a robust measure of feeding behavior released by the experimental stimulus being tested. Comparison of BPP rates over 30 second intervals achieved finer temporal resolution in description of the behavioral response thus certain data are plotted in this way.

Experiment 1: Is BPP rate of bigheaded carps increased by food mix presence? If so, are food mix induced BPP behaviors chemically mediated? If so, do the bigheaded carps have chemically based diet preferences?

Authors have deduced that chemical cues drive filter feeding behaviors in bigheaded carps (Beveridge, 1993; Dong and Li, 1994; Hansen et al., 2014; Smith, 1989). The first step towards characterizing the physiological basis of filter feeding behaviors is to test this hypothesis directly. Experiment 1 did this by changing the composition of the food mixture presented to fish to demonstrate the strong causal relationship between BPP

behavior and food/chemical food cue presence, quantify the role of the chemical senses in mediating the BPP response, and test for chemically based diet preferences.

Matched pre-stimulus observation intervals establish baseline respiratory BPP rates. The positive control for this experiment was food mixture. The negative control was well water. The nine other treatments were filtrates of food mixture ingredients prepared in identical concentrations as present in the food mixture.

Experiment 1 had 11 replicates of each of 12 treatments with silver carp, and 12 replicates of 12 treatments with bighead carp (12 days). Scoring of BPP rate was conducted by a single observer for silver carp and two observers for bighead carp. The observer recorded individual BPP events from one individual from each pair (the individual was initially randomly selected and then thereafter differentiated from the other by size) because simultaneous scoring of both fish in the pair was not possible.

Experiment 2: Does quality or quantity of chemical food cues modulate BPP rate?

Experiment 1 tested whether elevated BPP rates were evoked by food chemicals and whether certain components of the food mixture were more stimulatory than others, but could not discern the specific role of chemical composition (chemical food quality) because different components were present at different concentrations in this mixture. In Experiment #2, dilutions of chlorella and spirulina algae filtrates were tested in parallel using identical methods to Experiment #1. Chlorella and spirulina were selected because their filtrates produced markedly different responses in Experiment 1and represent taxonomically different phytoplanktonic food types (cyanobacteria and chlorophyte

algae). Experiment 2 had 10 replicates of each of 10 treatments with silver carp (9 days). The observer recorded individual BPP events from one individual from each pair (the individual was initially randomly selected and then thereafter differentiated from the other by size) because simultaneous scoring of both fish in the pair was not possible.

Experiment 3: Do common L-Amino acids explain chemically mediated BPP behavior?

Experiment 3 tested the activity of L-Amino acids alone as chemical feeding cues, because these chemical species are responsible for the majority of feeding behavior in model species of which this information is reported in the literature (Derby and Sorensen, 2008; Sorensen and Caprio, 1997; Valentincic and Caprio, 1994; Valentinčič and Caprio, 1997). The concentration of 18 free L-Amino acids in the food mix filtrate was quantified using high pressure liquid chromatography (HPLC) by Texas A&M Universities' Protein Chemistry Lab (Buha and Panchal, 2011). This information was used to create a synthetic mixture of free amino acids in well water that replicates the algal filtrate's proteinogenic L-Amino acid profile (Hansen et al., 2014). This solution was then tested alongside controls using the assay described above. L-Amino acids were obtained (Sigma Chemical; St. Louis, MO) and reconstituted in well water. The solution was agitated with a stir bar for 1 hour at room temperature and then stored in a refrigerator until use. Due to indeterminate quantification of Glu/Gln and Asp/Asn by HPLC, we mixed these species 50:50 respectively.

This experiment had 11 replicates of 3 treatments with silver carp (3 days), and 12 replicates of 4 treatments with bighead carp (3 days). The observer recorded individual

BPP events from one individual from each pair (the individual was initially randomly selected and then thereafter differentiated from the other by size) because simultaneous scoring of both fish in the pair was not possible.

Experiment 4: What is the role of the olfactory sense in BPP behavior?

To determine the physiological basis of BPP behavior, we blocked the olfactory system in Experiment #4. This procedure allowed us to directly examine the role of olfaction in previously described BPP responses to food stimuli. In this design each of 12 experimental replicates (6 *H. molitrix and* 6 *H. nobilis)* received the following trial sequence once per day at a random time 0830-1530 hours for nine days. First, combined BPP rates from each pair of fish over a 4 min period with no stimulus were scored. A 3ml volume of algal filtrate was then added via the remote stimulus delivery system described above and another 4 min response interval observed for BPP. Then, 3ml of algal food was added and another 4 min response interval observed for BPP. This procedure was repeated on each tank once per day. After the third day of testing was completed, 3M Express vinyl polysiloxane was inserted in the olfactory nares of the subject fishes (St. Paul, MN, USA) following established procedures (Levesque et al., 2011) but without anesthesia. Great care was taken to reduce stress on the fish through the <30 sec procedure. Half of the replicates received single nare occlusion (3 silver carp and 3 bighead carp) while the other half received dual nare occlusions (3 silver carp and 3 bighead carp). Occlusions were set with long tails to facilitate removal. Identical trial procedure and observations to that described above proceeded 15 hours after occlusion

set procedure for 3 more days. After 6th day of testing the occlusions were removed with fine forceps, followed by a further 3 days of identical trial procedure and observation. A total of 9 days of continuous data monitoring the effect of olfactory occlusion and then occlusion removal on the BPP responses to algal food filtrate and algal food. This experiment was recorded in high definition at 60 frames per second and scored *post-hoc* by 3 observers at a computer workstation using Noldus Observer XT software (version 11.5, Wageningen, NL). In this experiment data from both fish in each experimental replicate were scored and summed for analysis.

Analysis

Counts of buccal pumps were summated by observation interval and tagged with independent variables in Noldus Observer XT software, exported in comma delimited text format, and loaded into R for analysis (version 3.1.2,CRAN.r-project.org). Diagnostic plots and composite tests of normality revealed that data followed a log-normal distribution. Log transformations were performed on all data prior to statistical tests in order to meet assumptions of normality for ANOVA. ANOVA and Tukey's HSD methods were used to test for effects of design factors (Treatment, Species, Observer, Replicate Tank, Day, Time of Day, and the interaction between species and treatment) on the response variable at α=0.05 level. In experiments 1 and 3 identical tests were performed on each species on different sets of days. In these cases datasets from each species (identical test, different time) were combined for parsimonious analysis (unique day levels). A 3 parameter logistic growth model was fitted to dose response data series

in experiment 2 using package nlme (cran.r-project.org/package=nlme). Assay function was assessed for each experiment by using identical analysis on pre-test data, testing for effect of treatment under the logic that no effect should exist if the apparatus is working correctly.

Results

Experiment 1

The food mixture and food mix filtrate caused significant increases in BPP rate after addition ($F(1,410)=549.096$, $p<2.2e-16$). Mean baseline respiratory BPP rate over an 8 min pre-test period was 0.09 +/- 0.04 and 0.03 +/- 0.02 Hz for silver and bighead carp respectively across 12 pairs of fish. When food mixture was presented, mean BPP behavior increased to 2.44 +/- 0.25 and 1.97 +/- 0.48 Hz over an 8 min period for silver and bighead carp respectively and was sustained at an elevated rate for greater than 8 min. Chemical cues of the food mixture alone (food mix filtrate) released BPP rates of 1.11 +/- 0.59 and 1.15 +/- 0.45 Hz over an 8 min period for silver and bighead carp respectively. Responses to spirulina filtrate were strong in both species (similar to food mix filtrate); with a notable difference in potency of chlorella filtrate between species (Figure 2).

ANOVA found a statistically significant effect of food stimulus type ($F(11,151)=41.2885$, $p<2.2e-16$), and Tukey's HSD tests found evidence of chemically based food preferences that vary by species (Figure 3). ANOVA also found significant effects of replicate tank ($F(11,151)=3.5796$, $p=.0001$), experimental day

(F(24,151)=2.3845, p=.0008), species (F(1,151)=4.4642, p=.0363), and interaction between treatment and species (F(11,151)=4.1679, p=2.23e-05) on BPP behavior. Aside from demonstrating chemical food preferences, these data also bolster the conclusion that chemical cues are primarily mediating BPP behavior and suggest that the quality of chemical cues modulates the BPP response. Spirulina algae (*Arthrospira platensis*) released the relatively highest rates of BPP behavior between both silver and bighead carp (Figure 3).

Experiment 2

BPP responses to chlorella and spirulina filtrates showed a sigmoidal relationship over the log molar dilutions tested (Figure 4 Panel A). 100% Concentration of stimulus filtrate corresponded to the concentration found in the algal food mix. ANOVA found a significant effect of stimulus concentration (F(6,31)=12.3920, p=4.404e-07) and stimulus type (F(1,31)=9.0488, p=.005181). A three parameter logistic growth model was fitted to each of these data series, and plotted with a labelled upper asymptote (Figure 4 Panel B). By these models, BPP responses to spirulina filtrate are predicted to saturate at a much higher rate than BPP responses to chlorella, indicated by significantly different inflection point and upper asymptote (F(1,129)=16.58317, p=.0001; F(1,129)=99.98768, p<.0001). These results demonstrate that BPP responses of bigheaded carps are dependent on quality and quantity of chemical cues to which they are exposed.

Experiment 3

The mixture of 18 L-Amino acids produced a statistically significant BPP response which was also significantly less than the response to the food mix filtrate (Figure 5), indicating minor activity of the 18 L-Amino acids tested as part of the stronger BPP response to whole algal food filtrate. ANOVA found a statistically significant effect of treatment in test data ($F(2,29)=64.8272$, $p=1.987e-11$), and a statistically significant effect of experimental day ($F(7,29)=5.1343$, $p=.0008$). ANOVA found no significant effect of species ($F(1,29)=.0378$,$p=.8471$).

Experiment 4

The results of the olfactory occlusion experiment suggest an active role of olfaction in modulating far-field feeding behaviors and of both olfaction and gustation in controlling near-field feeding behaviors (Figure 6). ANOVA found significant effect of tank ($F(11,304)=4.1277$, $p=1.115e-05$), stimulus type ($F(2,304)=448.3072$, $p<2.2e-16$), and occlusion state ($F(3,304)=33.9862$, $p<2.2e-16$), but found no effect of species ($F(1,304)=.0743$, $p=.7854$) or an observer effect for either subject in the pair ($F(1,304)=.0015$, $p=.9693$;$F(1,304)=.0238$, $p=.8774$). Tukey's HSD tests found significant differences between pre-occlusion baseline state, single and double occlusion states, and post-occlusion recovery state; but not between single and double occlusion states (Figure 6 panel B).

Discussion

This study is the first to demonstrate the important role that chemical cues play in the control of filter feeding behaviors of microphagous fishes and the genus *Hypophthalmichthys*. We found that a particulate food mixture was discerned by its chemical components with certain components having a more substantial role in releasing characteristic filter feeding behavior than others. Differences in chemically based food preferences between silver and bighead carp were statistically significant. Despite being tested at lab scale, the authors believe that these data fundamentally reflect behavior patterns of feral bigheaded carp. As demonstrated multiple times in different ways, chemical food cues alone produce an intense filter feeding response in the silver and bighead carp that approaches the potency of the entire suite of food cues for short periods.

The behavior of bigheaded carps (*Hypophthalmichthys spp.*) in Experiments #1-4 strongly suggests that microphagous fish primarily rely on their olfactory systems to perceive/find preferred food items and on their gustatory epibranchial/gill raker cross-flow filter apparatus to sample and ingest/reject their diet of micro-particulate food items (similar sensory involvement to channel catfish, rainbow trout, and goldfish). The suite of chemical cues released by a particular food type is perceived as an identifying signal of the quality and quantity of the food; stimulating feeding behavior of the appropriate magnitude (Derby and Sorensen, 2008; Lindstedt, 1971). The significant reduction in BPP response to food mix filtrate after olfactory occlusion signals a predominant role of olfaction in far-field perception of food particles in the immediate environment. The

gustatory and/or solitary chemosensory cell senses appear to be involved with mediating reflexive intra-oral ingestion behavior (sustained rhythmic BPP)(Hansen et al., 2014). We interpret these data to mean that olfaction is important for the far-field stages of filter feeding behaviors (perception, search, and sampling) while gustation, being closely coupled with reflexive near field and intra-oral elements of BPP behaviors (ingestion), can partially fill this role and "jump-start" filter feeding behavior sequence in anosmic bigheaded carp.

The other sensory modalities not directly studied may also have roles in sensation and perception of food when available, e.g. visual, lateralis, and tactile senses. Evidence for non-chemical sensory involvement is found in elevated BPP rates stimulated by food mix filtrate being suppressed far quicker than the chemical food cues were diluted in our test environment (Appendix 2), suggesting that along with primary olfactory and gustatory control of BPP behavior an additional sensory feedback loop exists to judge the actual consumption of food particles in relation to BPP effort and modulate accordingly. The mechanism of food particle perception is undefined but the sensory field is likely located in/on the epibranchial/gill raker filtration apparatus, in the epithelium around the pharyngeal teeth, or inside the foregut. For this reason, we postulate that the physiological basis of this feedback loop is some combination of tactile and chemical senses, possibly functioning in a similar manner to bimodal human wetness detection (Filingeri et al., 2014). Regardless of the physiological basis, data show that when the food particles are produced from BPP behavior effort the duration of the behavior is extended. In this way, the sampling component of bigheaded carp feeding behavior (BPP)

appears to be released by chemosenses and stimulated/suppressed by this yet undefined system (Lindstedt, 1971).

Our investigation into the role of free aqueous L-Amino acids as odorants or tastants stimulating BPP behavior led to an unexpected conclusion. Free amino acids are well described as important odorants and tastants for many species of fishes, and usually account for all of the behavioral or electrophysiological activity of a preferred food (reviewed by Derby and Sorensen, 2008). For this reason we hypothesized a strong role of this class of molecules in the BPP responses of bigheaded carps. Our results suggest otherwise; specifically that for these species there are other chemical species present in the algal food filtrate that are functioning as the primary odor/taste object and/or as part of a complex chemical cue mixture.

The structural identities of these chemical cues are not yet defined, but this initial body of research has guided how this information may be obtained. Future work on the subject will apply similar behavioral methods and more advanced analytical chemistry techniques in order to identify the structural identities of the active chemical food cues mediating BPP behavior through bioassay guided fractionation (Sorensen and Hoye, 2007), assess broader diet preferences, and to investigate the role of associative conditioning on the BPP response.

We postulate that BPP rate is a viable metric to assess food preferences in certain fishes at lab scale, as opposed to electivity indices (Kolar et al., 2005; Lazzaro, 1987), which have major confounding issues (O'Brien and Vinyard, 1974). This study found spirulina (*Arthrospira spp.*) to be a chemically preferred food type among the ingredients

of our lab's maintenance diet to both species of bigheaded carp studied. This is interesting and relevant because it is well established that the bigheaded carps readily consume cyanobacteria as adults (Beveridge, 1993; Ye et al., 2013) and that cyanobacteria produce unique suites of chemical cues compared to green algae , lending to the taxon's potential to function as a genus-specific food bait.

While generalized responses were similar, silver carp displayed elevated rates of respiratory and filter feeding BPP behavior compared to bighead carp. Experiment 1 data also detected species differences in their chemically based food preferences, suggesting that species specific chemical food preferences exist in our lab populations and therefore likely exist in feral populations; consistent with consensus from study of gut contents and stable isotope analyses.

Assuming that a bigheaded carp specific chemical feeding attractant/stimulant formulation exists and can be characterized, quantified, and produced at large scale, it could be eventually used in conjunction with several traditional and modern methods under development and use by United States federal and state government agencies to control feral bigheaded carps. Chemical feeding cues could be actively applied to enhance the efficiency of selective poisoning and/or traditional removal techniques or passively monitored to predict where and when aggregations of feral carp will form.

Knowledge of the chemical ecology and feeding preferences of these species could also be applied to bolster production rates and/or enhance pharmaceutical delivery efficiency in aquaculture, as the bigheaded carps are commercially important at a global scale (Michielsens and Lorenzen, 2002). This same knowledge may also guide

management decisions in native ranges where they are increasingly threatened by habitat loss. Most broadly, the bigheaded carps now serve as the first model for understanding the physiological basis of feeding behavior in microphagous fish taxa.

Figures

Figure 1.) Mass balance of the algal food mix when in well water solution. Chemical cues released after ingredients are mixed and added to well water were 1.46 g/L. Color palette for ingredients is consistent throughout all figures.

Figure 2.) Mean buccal-pharyngeal pumping (BPP) rates of subject fish in response to selected food treatments from Experiment 1 plotted at 30 second time intervals (*H.molitrix* n=11, *H.nobilis* n=12). Food stimuli were added after 8 minutes of baseline recording.

Figure 3.) BPP rates of pairs of subject fish in response to filtrates of 9 ingredients of the algal food mix plotted over 8 min time intervals (H.molitrix n=11, H.nobilis n=12) in comparison to control treatments (well water, food mix, and food mix filtrate). Responses are ranked by mean (shown at top) and results of Tukey's HSD are depicted by lines connecting treatments that are not significantly different (bottom).

Figure 4.) A dose response assay (Experiment 2) was conducted using spirulina and chlorella filtrates, two treatments that produced significantly different BPP responses in Experiment 1 and 2 for silver carp (Panel A). 100% Concentration corresponds to the concentration by dry mass found in the algal food mix. A 3 parameter logistic growth

model was fitted to each of these data series and plotted with asymptotes and predicted values for reference (Panel B).

Figure 5.) BPP rates of pairs of subject fish in response to a mixture of 18 L-Amino acids replicating the profile of algal food mix filtrate plotted over 8 minute time intervals (*H.molitrix* n=11, *H.nobilis* n=12) in comparison to control treatments (well water, food mix, and food mix filtrate). Responses are sorted by median to facilitate ranking, and results of Tukey's HSD are depicted by lines connecting treatments that are not significantly different.

Figure 6.) Effects of olfactory occlusion on the BPP response to well water, food mix filtrate, and food mix in bigheaded carps (Panel A and B). The BPP behavior of 12 pairs of subject fish (6 silver carp *H.molitrix*, 6 bighead carp *H.nobilis*; 24 total) was quantified over a 9 day period in which they received one 12 minute trial per day consisting of the following events in sequential order: 1.) injection of blank (nothing) and 4 minute observation, 2.) injection of 3ml food mix filtrate and 4 minute observation, and 3.) injection of 3ml food mix and 4 minute observation. After observation on day 3 was completed, olfactory occlusions were installed (6 replicates had 1 olfactory nare occluded - hyposmic, 6 replicates had 2 olfactory nares occluded - anosmic; balanced systematically between species). After observation on day 6 was completed, olfactory occlusions were removed. Results of Tukey's HSD tests between occlusion states are depicted by lines connecting factor levels that were not significantly different (Panel B).

The potential confounding effect of procedural stress is internally controlled in this experiment by the immediate recovery of response after occlusion removal.

Figure 1.)

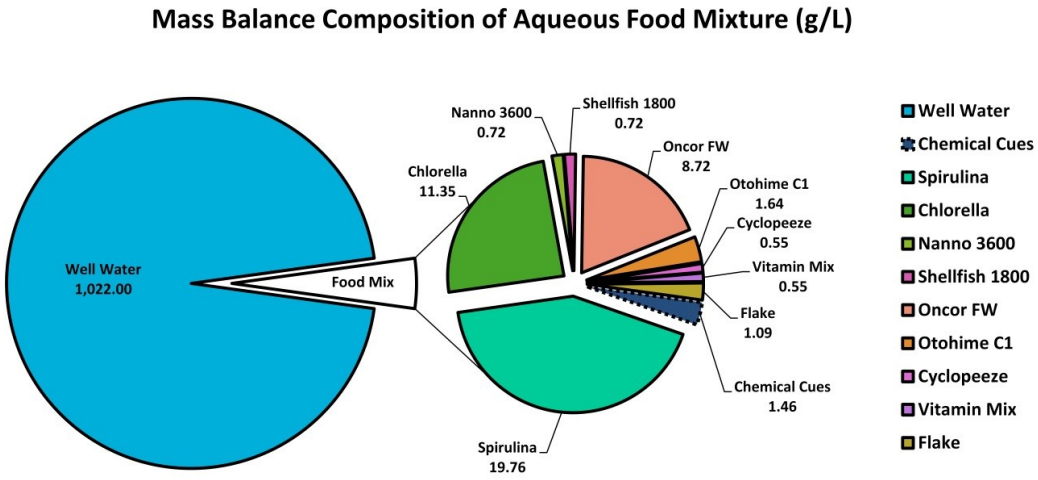

Mass Balance Composition of Aqueous Food Mixture (g/L)
Well Water
1,022.00
Food Mix
Nanno 3600
0.72
Shellfish 1800
0.72
Oncor FW
8.72
Chlorella
11.35
Otohime C1
1.64
Cyclopeeze
0.55
Vitamin Mix
0.55
Flake
1.09
Chemical Cues
1.46
Spirulina
19.76
Well Water
Chemical Cues
Spirulina
Chlorella
Nanno 3600
Shellfish 1800
Oncor FW
Otohime C1
Cyclopeeze
Vitamin Mix
Flake

Figure 2.)

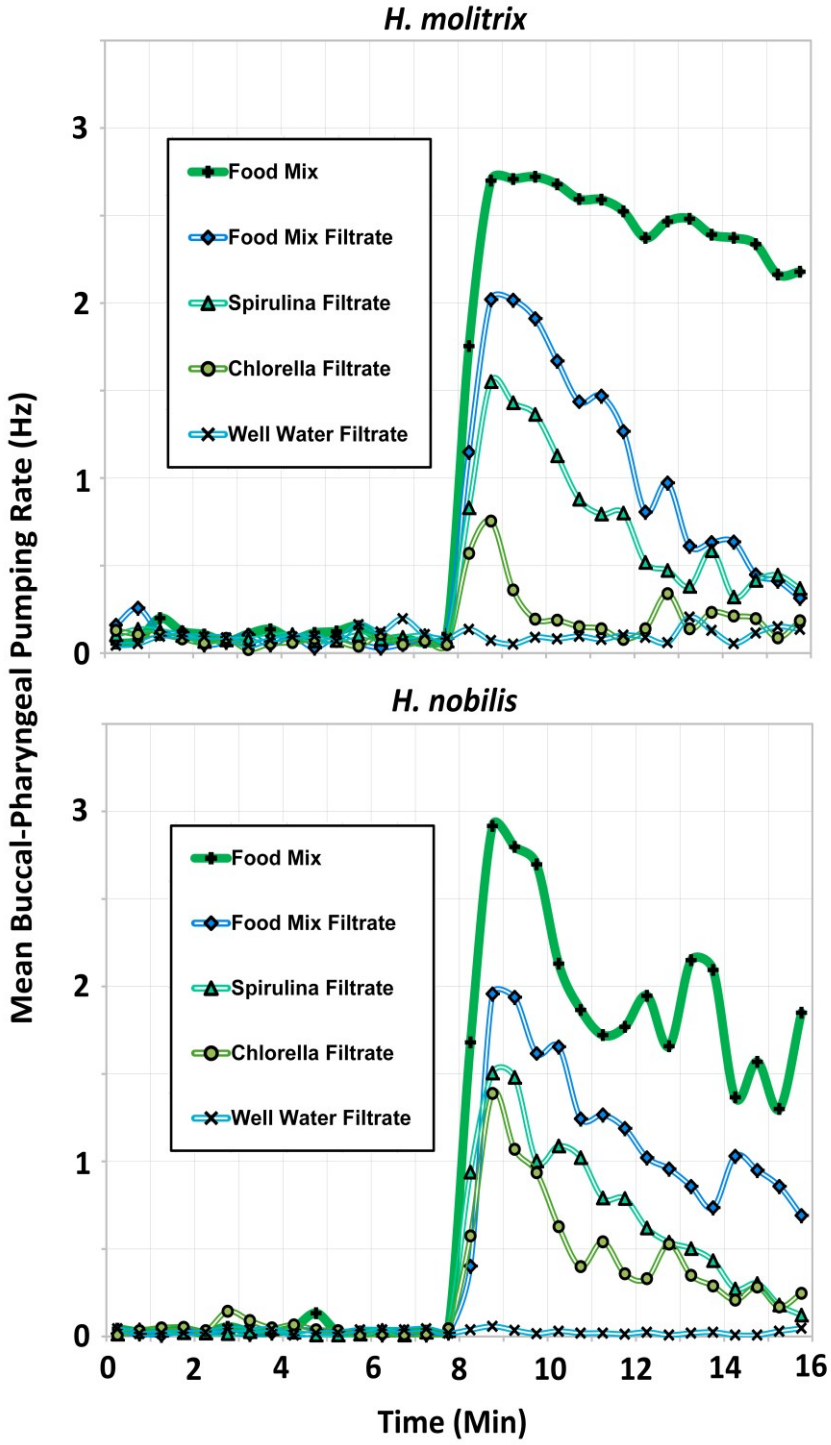

H. molitrix
H. nobilis
Food Mix
Food Mix Filtrate
Spirulina Filtrate
Chlorella Filtrate
Well Water Filtrate
Mean Buccal-Pharyngeal Pumping Rate (Hz)
Time (Min)
0
1
2
3
0
2
4
6
8
10
12
14
16

Figure 3.)

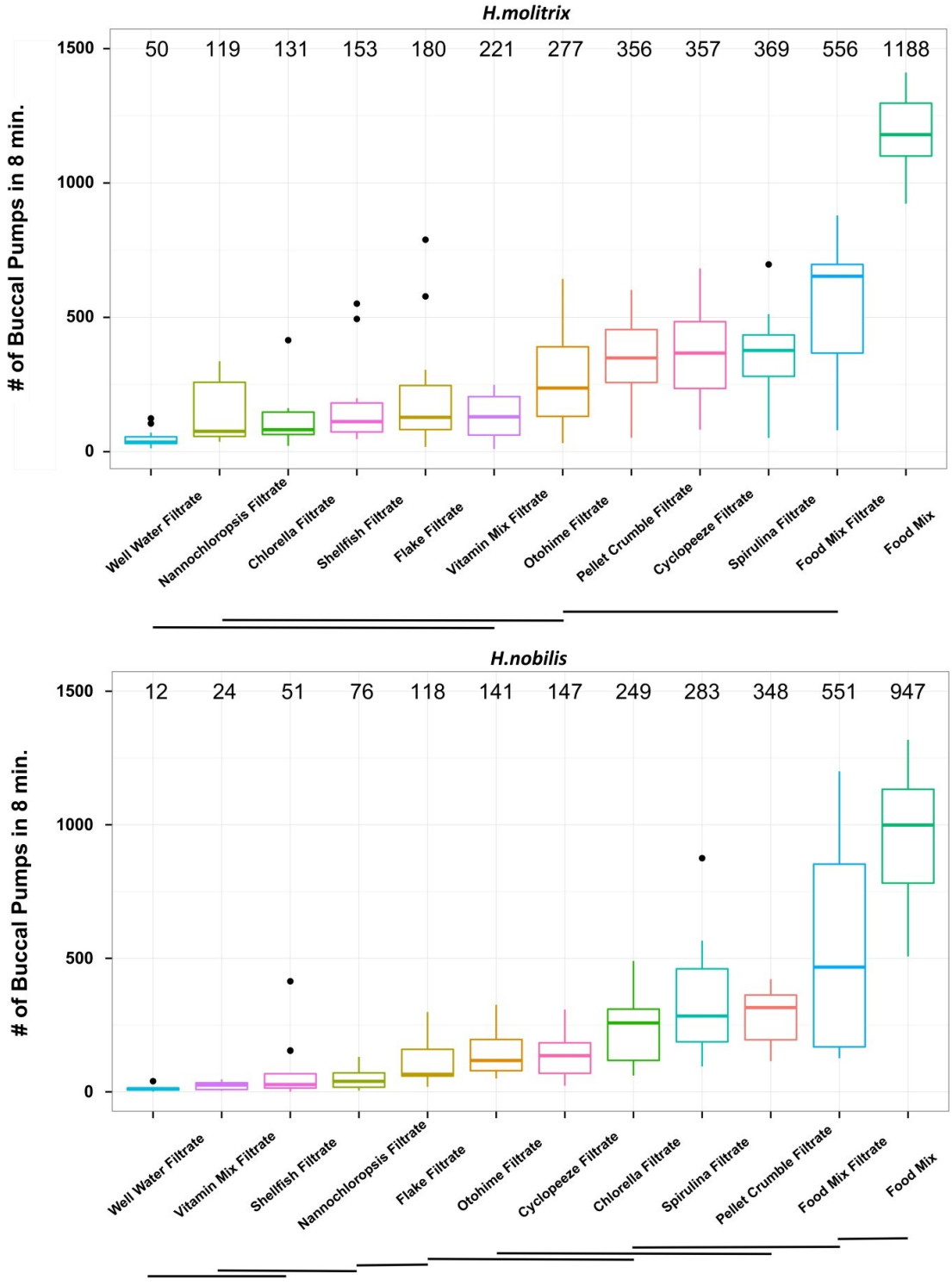
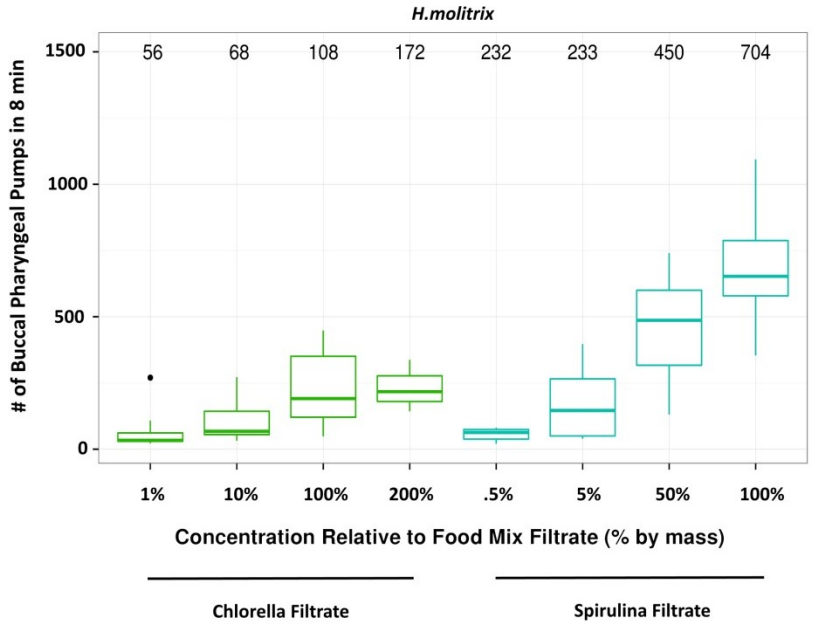

H.molitrix
# of Buccal Pumps in 8 min.
1500
1000
500
0
50
119
131
153
180
221
277
356
357
369
556
1188
Well Water Filtrate
Nannochloropsis Filtrate
Chlorella Filtrate
Shellfish Filtrate
Flake Filtrate
Vitamin Mix Filtrate
Otohime Filtrate
Pellet Crumble Filtrate
Cyclopeeze Filtrate
Spirulina Filtrate
Food Mix Filtrate
Food Mix
H.nobilis
# of Buccal Pumps in 8 min.
1500
1000
500
0
12
24
51
76
118
141
147
249
283
348
551
947
Well Water Filtrate
Vitamin Mix Filtrate
Shellfish Filtrate
Nannochloropsis Filtrate
Flake Filtrate
Otohime Filtrate
Cyclopeeze Filtrate
Chlorella Filtrate
Spirulina Filtrate
Pellet Crumble Filtrate
Food Mix Filtrate
Food Mix

Figure 4.)


H.molitrix
# of Buccal Pharyngeal Pumps in 8 min
1500
1000
500
0
56
68
108
172
232
233
450
704
1%
10%
100%
200%
.5%
5%
50%
100%
Concentration Relative to Food Mix Filtrate (% by mass)
Chlorella Filtrate
Spirulina Filtrate

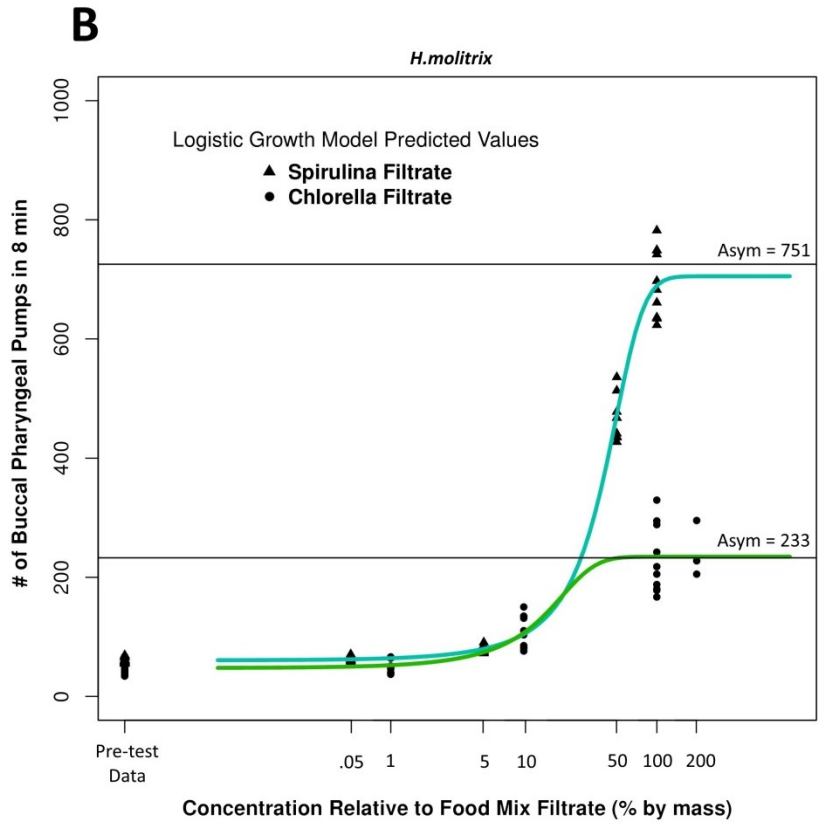

B
H.molitrix
# of Buccal Pharyngeal Pumps in 8 min
1000
800
600
400
200
0
Logistic Growth Model Predicted Values
Spirulina Filtrate
Chlorella Filtrate
Asym = 751
Asym = 233
Pre-test Data
.05
1
5
10
50
100
200
Concentration Relative to Food Mix Filtrate (% by mass)

Figure 5.)

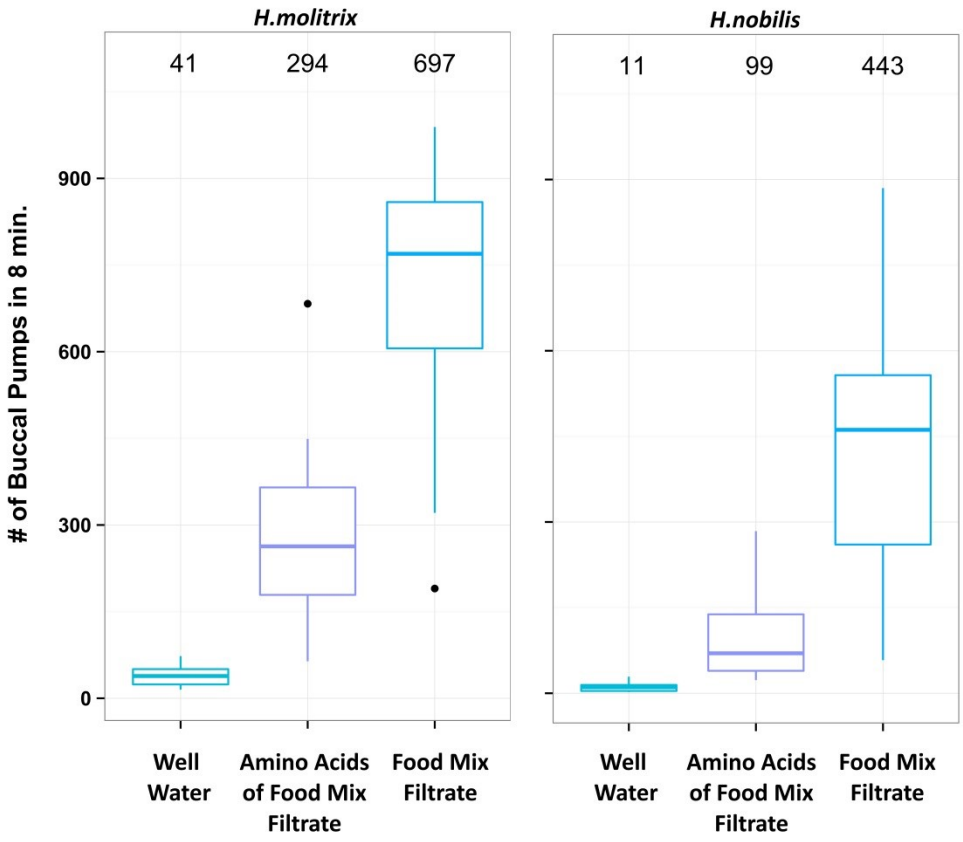
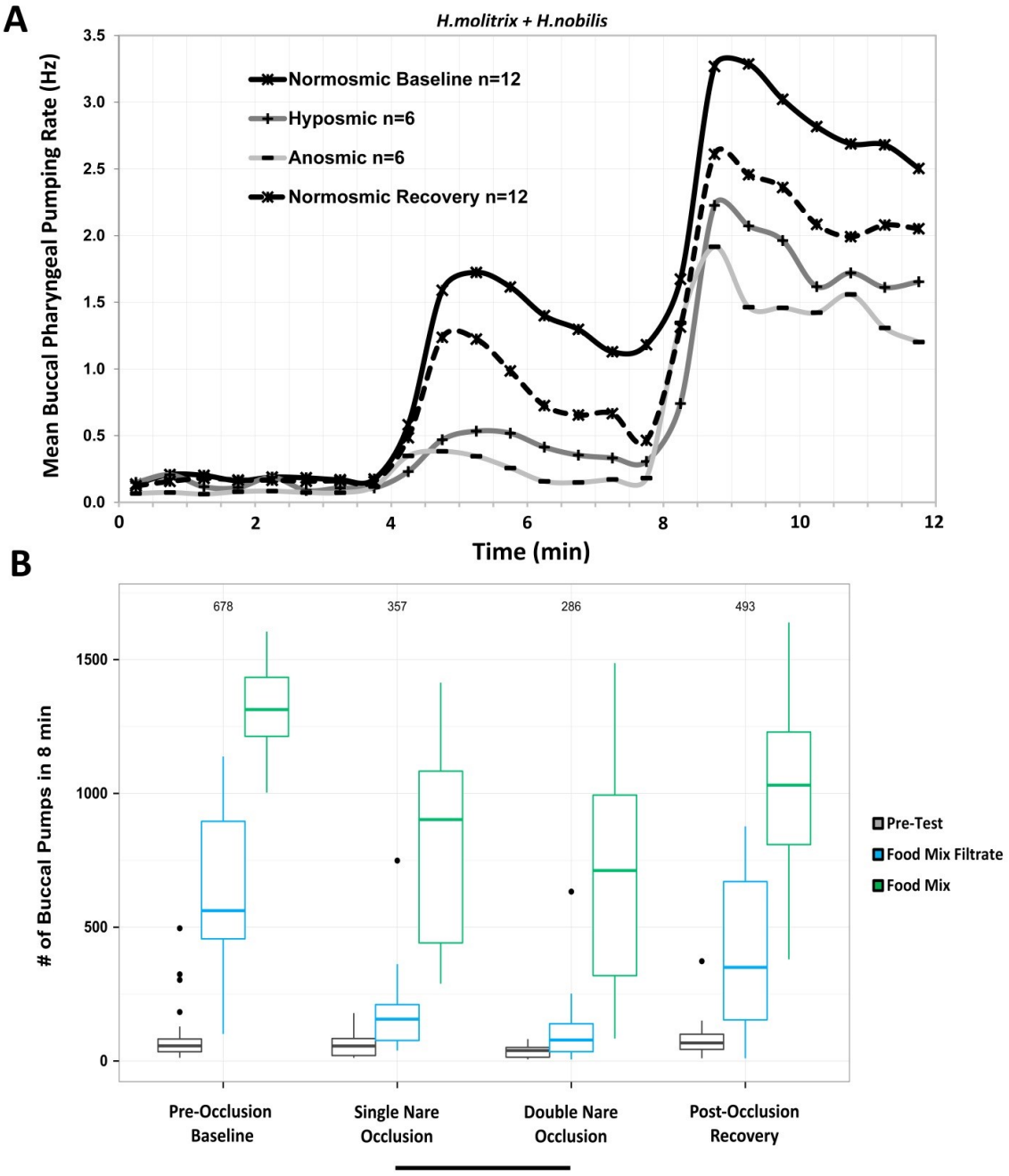

H.molitrix
H.nobilis
41
294
697
11
99
443
# of Buccal Pumps in 8 min.
900
600
300
0
Well Water
Amino Acids of Food Mix Filtrate
Food Mix Filtrate
Well Water
Amino Acids of Food Mix Filtrate
Food Mix Filtrate

Figure 6.)


A
H.molitrix + H.nobilis
Normosmic Baseline n=12
Hyposmic n=6
Anosmic n=6
Normosmic Recovery n=12
Mean Buccal Pharyngeal Pumping Rate (Hz)
Time (min)
B
678
357
286
493
# of Buccal Pumps in 8 min
Pre-Test
Food Mix Filtrate
Food Mix
Pre-Occlusion Baseline
Single Nare Occlusion
Double Nare Occlusion
Post-Occlusion Recovery

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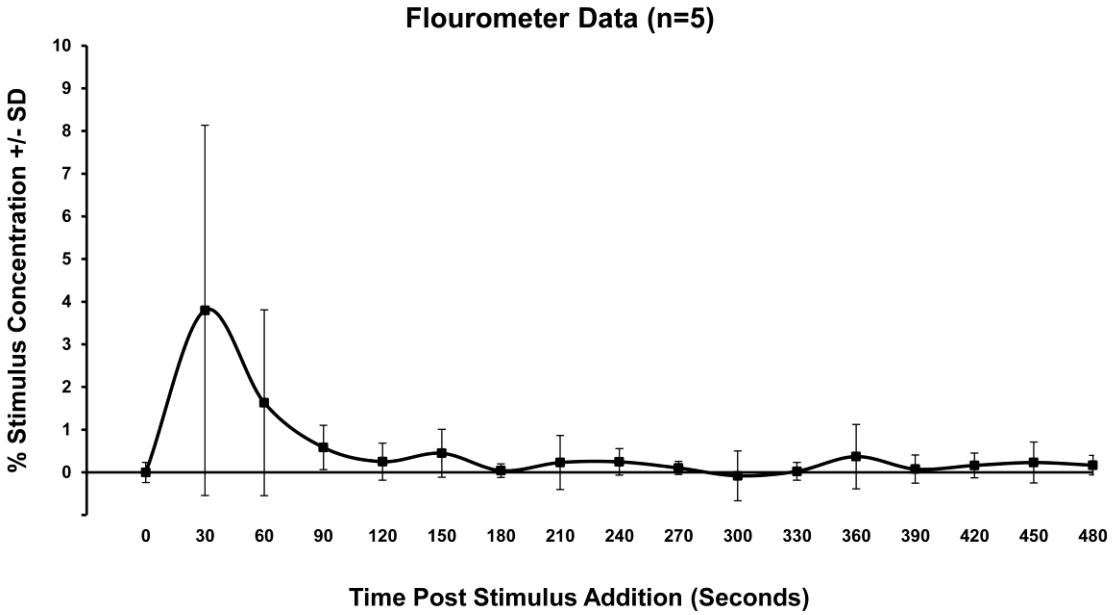
Appendix 1: Hybrid Genetics of Bigheaded Carps used in Behavioral Experiments

A fin tissue sample was taken from each of 20 randomly chosen individual fish from each group used in behavior experiments. Samples were analyzed by James Lamer for hybridization using published methods developed for these species using mitochondrial (mt-C0I) and nuclear (57 Single Nucleotide Polymorphisms) DNA markers (Lamer et al., 2013). This analysis revealed considerable levels of introgression in our lab fish populations. The silver carp group used in this study contained 47% post-f1 hybrids (n=20), while our bighead cohort contained 30% post-f1 hybrids (n=20). There were no first generation hybrids sampled; most of the hybrids in both groups were 3rd or 4th generation backcrossed individuals. These population genetics are consistent with populations of the upper Mississippi river basin (Lamer and Dolan, 2010; Lamer et al., 2013).

Appendix 2: Quantification of Stimulus Dilution Rate in Test Tanks

Rhodamine dye (Turner Designs, Sunnyvale, CA) and an Aquaflour™ fluorimeter (Turner Designs, Sunnyvale, CA) were used to measure the dilution of chemical stimuli into the 70 liter test tank. The fluorimeter was calibrated using well water from the tank as the blank sample and 100 ppb dye solution as the reference sample. In each of 5 trials, 3 ml of 100 ppb dye solution was added to the test tank with the pneumatic Pasteur pipette system in the same manner in which stimuli are added during behavior experiments; after which 3 ml water samples were taken using a 5 ml pipette (Oxford Instruments, Oxfordshire, UK) from the center of the tank every 30 seconds for eight minutes.

Data reflect show a biphasic dilution pattern with high variance (Appendix 2 Figure 1, below). Stimuli are mostly homogeneous in the test tank after 90 seconds. In behavioral trials, fish are least likely to encounter the stimulus before it is diluted 10 fold, most likely to encounter the stimulus when it has been diluted greater than 10 fold, and certain to encounter it before it is diluted 23,333 fold. Low concentrations of stimuli remain in the tank for the duration of the 8 minute test period. These data suggest that dilution alone cannot explain the extinction of BPP behavior in Experiment #1.



Appendix 2 Figure 1. Time-series fluorimeter data from the 70 liter test tanks used in behavioral experiments.